Chlamydiae in febrile children with respiratory tract symptoms and age-matched controls, Ghana

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Abstract: Members of the Chlamydiales order are obligate intracellular pathogens causing acute and chronic infectious diseases. Chlamydiaceae are established agents of community- and zoonotically acquired respiratory tract infections, and emerging pathogens among the Chlamydia-related bacteria have been implicated in airway infections. The role of both in airway infections in Africa is underexplored. We performed a case -control study on the prevalence of Chlamydiaceae and Chlamydia-related emerging pathogens in children with febrile respiratory tract infections in West Africa, Ghana. Using a pan-Chlamydiales broad-range real-time PCR, we detected chlamydial DNA in 11 (1.9%) of 572 hospitalized febrile children with respiratory tract symptoms and in 24 (4.3%) of 560 asymptomatic age-matched controls (p 0.03). Chlamydiaceae were found to be common among both symptomatic and healthy Ghanaian children, with Chlamydia pneumoniae being the most prevalent species. Parachlamydiaceae were detected in two children without symptoms but not in the symptomatic group. We identified neither Chlamydia psittaci nor Simkania negevensis but a member of a new chlamydial family that shared 90.2% sequence identity with the 16S rRNA gene of the zoonotic pathogen Chlamydia pecorum. In addition, we found a new Chlamydia-related species that belonged to a novel family sharing 91.3% 16S rRNA sequence identity with Candidatus Syngnamydia venezia. The prevalence and spectrum of chlamydial species differed from previous results obtained from children of other geographic regions and our study indicates that both, Chlamydiaceae and Chlamydia-related bacteria, are not clearly linked to clinical symptoms in Ghanaian children.

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ORIGINAL ARTICLE

Chlamydiae in febrile children with respiratory tract symptoms and age-matched controls, Ghana

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Abstract

Members of the *Chlamydiales* order are obligate intracellular pathogens causing acute and chronic infectious diseases. *Chlamydiaceae* are established agents of community- and zoonotically acquired respiratory tract infections, and emerging pathogens among the *Chlamydia*-related bacteria have been implicated in airway infections. The role of both in airway infections in Africa is underexplored. We performed a case -control study on the prevalence of *Chlamydiaceae* and *Chlamydia*-related emerging pathogens in children with febrile respiratory tract infections in West Africa, Ghana. Using a pan-*Chlamydiales* broad-range real-time PCR, we detected chlamydial DNA in 11 (1.9%) of 572 hospitalized febrile children with respiratory tract symptoms and in 24 (4.3%) of 560 asymptomatic age-matched controls (p 0.03). *Chlamydiaceae* were found to be common among both symptomatic and healthy Ghanaian children, with *Chlamydia pneumoniae* being the most prevalent species. *Parachlamydiaceae* were detected in two children without symptoms but not in the symptomatic group. We identified neither *Chlamydia psittaci* nor *Simkania negevensis* but a member of a new chlamydial family that shared 90.2% sequence identity with the 16S rRNA gene of the zoonotic pathogen *Chlamydia pecorum*. In addition, we found a new *Chlamydia*-related species that belonged to a novel family sharing 91.3% 16S rRNA sequence identity with *Candidatus* Syngnamydia venezia. The prevalence and spectrum of chlamydial species differed from previous results obtained from children of other geographic regions and our study indicates that both, *Chlamydiaceae* and *Chlamydia*-related bacteria, are not clearly linked to clinical symptoms in Ghanaian children.

Keywords: Children, *Chlamydia*, *Chlamydia*-related bacteria, febrile respiratory tract infection, Ghana
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Introduction

Members of the order *Chlamydiales* are strictly intracellular pathogens featuring a biphasic developmental cycle. *Chlamydiaceae* are responsible for many acute and chronic diseases in humans and animals. In the context of respiratory tract infections, *Chlamydia pneumoniae* and *Chlamydia psittaci* are established pathogens causing community- and zoonotically

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acquired pneumonia, respectively [1]. *Chlamydia trachomatis* is a major cause of sexually transmitted diseases and can cause pneumonia in neonates if transmitted during birth [2]. *Chlamydia*-related bacteria are emerging pathogens; amongst others, *Protochlamydia, Waddlia* and *Simkania* have been considered to be responsible for respiratory tract infections in humans, the latter especially in children [3–5].

The prevalence of respiratory tract infections associated with *C. pneumoniae* has been reported to range from 0 to 44.2%, depending on the population, the age distribution, the geographic region studied and the diagnostic methods used [6]. In recent studies, a decrease in respiratory tract infections caused by *C. pneumoniae* was reported in the setting of industrial countries [7,8].

Little is known about the role of chlamydiae in respiratory tract infections in Africa. So far, only *C. pneumoniae* and *C. trachomatis* have been considered in studies on aetiologic agents of airway infections in African patients. Among refugees with respiratory infections who originated predominantly from Somalia and Sudan and who lived in Kenyan and Djiboutian refugee camps, 3.8% and 5.2% were found to be positive for *C. pneumoniae,* respectively [9,10]. In South Africa in the 1980s, *C. pneumoniae* was associated with 20.7% of community-acquired pneumonia in adults [11]. A study conducted in Alexandria and neighbouring rural areas in 2014 revealed the presence of *C. pneumoniae* in 31.4% of febrile children with respiratory infections using diagnostic real-time PCR [12]. Moreover, among 100 South African children with lower respiratory tract symptoms, 6% were reported to have *C. trachomatis* infections [13].

The aim of this study was to search for *Chlamydiaceae* and *Chlamydia*-related emerging pathogens among hospitalized febrile children with respiratory tract symptoms and age-matched fever-free controls from Ghana in West Africa.

Materials and methods

From November 2013 to September 2015, oropharyngeal swabs (flocked swabs; Copan Industries, Brescia, Italy) were collected from 572 children older than 1 month and younger than 15 years with a tympanic temperature of $\geq$38°C admitted to the paediatric ward of Agogo Presbyterian Hospital, a district hospital with 250 beds situated in the Asante Akim North municipality, Ghana. The admitted children presented either symptoms of the lower (i.e. cough, intercostal retractions, chest indrawings or nasal flaring; n = 522) or upper (i.e. blocked nose, coryza; n = 50) respiratory tract.

Additionally, oropharyngeal swabs from 560 fever-free asymptomatic children younger than 15 years of age with a temperature of <37.5°C without signs of a respiratory tract infection were recruited at vaccination clinics surrounding Agogo Presbyterian Hospital from September 2014 to September 2015. Briefly, DNA was extracted in Ghana using the Stratec Molecular RTP Pathogen Kit (Stratec Biomedical, Birkenfeld, Germany) and frozen at −80°C. We then performed a broad range pan-*Chlamydiales* 16S rRNA real-time PCR, as described elsewhere [3]. As a positive control, we used plasmid pCR2.1-TOPO (Thermo Scientific Fisher, Rheinach, Switzerland) which contained a portion of the 16S rRNA gene targeted by the pan-*Chlamydiales* 16S rRNA real-time PCR and genomic DNA from *C. trachomatis* D/UW-3/Cx. Moreover, internal amplification (RT-IPCY-B02; Eurogentec, Seraing, Belgium), extraction and no template controls were performed. Samples with a cycle threshold of $\leq$37 were considered positive.

After transport on dry ice to Europe, the 16S rRNA pan-*Chlamydiales* real-time PCR results were confirmed, and all samples with a cycle threshold of $\leq$35 were subjected to a complementary *Chlamydiaceae*-specific 23S rRNA real-time PCR [14]. The *Chlamydiaceae*-specific real-time PCR included internal amplification (using primers EGFP-1-F and EGFP-10-R [15]), positive (using genomic DNA from *Chlamydia abortus*) and no template controls. Additionally, samples with a cycle threshold of $\leq$35 in the 16S rRNA pan-*Chlamydiales* real-time PCR were analysed by sequencing the 16S rRNA amplicon. Practically, the best BLAST hit (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the obtained sequence with a 16S rRNA sequence from a bacterial strain that has been previously assigned taxonomically to a given species-level lineage was considered the first known organism in the National Center for Biotechnology Information (NCBI) database, and the percentage of similarity with that hit was used to perform the taxonomic assignment, as previously described [3,16–18]. Cutoffs of $\geq$80% [19], $\geq$92.5% [20], $\geq$95% [21] and $\geq$97% [22] sequence identity of the amplicon were used to assign the order, family, genus and species level, respectively. Short sequences (in the range of 97–108 bp) were assigned at the genus level if sequence identities of $\geq$95% were found, and at the family level if sequence identities of $\geq$92.5% and <95% were found.

In the statistical analysis, categorical variables were described as frequencies and percentages. Continuous variables were described using medians and their corresponding interquartile ranges (IQRs). To display age effects, four age categories (0, 1, 2–4 and >4 years) were established. The chi-square test was used to compare means, with $p < 0.05$ being considered statistically significant. All data analyses were performed by Stata 14 (StataCorp, College Station, TX, USA). The Committee on Human Research, Publications and Ethics, School of Medical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana, and the Ethikkommission der Ärztekammer Hamburg, Hamburg, Germany, provided ethical approval for this study. All participants were informed about the study's purpose and procedures.

TABLE 1. Demographic characteristics of study participants

Characteristic	Total (n = 1132)	Symptomatic cases (n = 572)	Controls (n = 560)
Sex (% female)	553 (48.9)	263 (46.0)	290 (51.8)
Age (months), median (interquartile range)	23 (12–47)	24 (12–44)	23 (13–47)
Age category, n (%)			
0 years	272 (24.0)	133 (23.3)	139 (24.8)
1 year	348 (30.7)	141 (24.7)	207 (37.0)
2–4 years	253 (22.4)	176 (30.8)	77 (13.5)
>4 years	259 (22.9)	122 (21.3)	137 (24.5)

Written informed consent was obtained from parents or guardians on behalf of the study children before study enrolment.

Results

We screened oropharyngeal swabs from 572 febrile children with respiratory tract symptoms and from an age-matched symptomless control group of 560 afebrile children from Ghana. Age was distributed similarly between cases (median, 24 months; IQR, 12–44 months) and controls (median, 23 months; IQR, 13–47 months) (Table 1). Girls were slightly underrepresented among cases (46.0%) and overrepresented among controls (51.8%). Using a pan-*Chlamydiales* broad-range real-time PCR [3], we found chlamydial DNA in 11 (1.9%, mean cycle threshold 34.1±2.7) febrile children with respiratory tract symptoms and in 24 (4.3%, mean cycle threshold 35.3±1.6) asymptomatic children (total n = 35), with the microorganism being significantly more frequent among the control group (p 0.03). With a median age of 23 months (IQR, 16–36) *Chlamydiales*-positive children did not exhibit any different age distribution among groups. No temporal patterns or seasonalities were observed.

Among the 35 *Chlamydiales*-positive samples, sequencing of the 16S rRNA amplicons and/or real-time PCR targeting the 23S rRNA gene allowed us to classify 15 chlamydiae-positive samples to the family (n = 5), genus (n = 2) or species (n = 8) level (Table 2). The phylogenetically characterized samples (n = 15) originated from six febrile (three boys, three girls) and nine healthy children (five boys, four girls). In four febrile children aged 1 to 4 years, partial 16S rRNA sequences (range, 135–206 bp) showed 100% sequence identity to *C. pneumoniae*. In one 3-year-old boy, the retrieved sequence was too short (108 bp) to determine the chlamydial species but could be assigned to the genus *Chlamydia*. A novel *Chlamydia*-related species belonging to an as-yet unclassified family was detected in a sample of a symptomatic 1-year-old girl. All six children had body temperatures between 38.3°C and 40°C, and had a cough (n = 5) and/or a runny or blocked nose (n = 4). Children in the control group with positive PCR results (n = 9) were between 11 months and 10 years old, and they were clinically healthy, with body temperatures ranging from 36.2°C to 37.4°C. Sequencing revealed partial 16S rRNA sequences with 100% sequence identity with *C. pneumoniae* (n = 3) and *C. trachomatis* (n = 1). In the other five cases, chlamydial determination at the species level was not possible, but sequences could be grouped into the genus *Protochlamydia* (n = 1), the family *Parachlamydiaceae* (n = 1) or *Chlamydiaceae* (n = 2), or into a new family of the order *Chlamydiales* (n = 1).

Discussion

Epidemiologic studies on the causative role of pathogens in human diseases are frequently limited by the lack of a respective equally sized, age-matched control group, in particular when evaluating emerging pathogens as an aetiologic agent. We conducted what is to our knowledge the largest case–control study on the prevalence of pathogenic *Chlamydiaceae* and emerging pathogens among *Chlamydia*-related bacteria in children with febrile respiratory tract infections. Compared to a previous study from Switzerland including children with (n = 265) and without pneumonia (n = 157) [3], our work revealed an almost fourfold lower level of exposure to *Chlamydiales* among Ghanaian children (11.4% in Switzerland vs. 3.1% in Ghana). In contrast to the Swiss study, where *Chlamydiaceae* were almost exclusively detected in ill children, we found *Chlamydiaceae* to be abundant among Ghanaian children both with and without respiratory tract infection. The most prevalent species identified in this study was *C. pneumoniae*. This respiratory tract pathogen was found in four of six and three of nine phylogenetically characterized samples from case and control patients, respectively.

C. pneumoniae has been described to cause asymptomatic infections in clinically healthy patients [23,24], and our results indicate the presence of such subclinical infections or colonization with *C. pneumoniae* in the control group. The children included in our study are, at least in the neighbouring rural area of Agogo, in close contact with poultry and other livestock that are implicated in the zoonotic transmission of *C. psittaci* to humans. Nevertheless, we did not detect *C. psittaci* but found DNA of a member of a new chlamydial family sharing 90.2% sequence identity with the 16S rRNA sequence of *C. pecorum*, a well-known animal pathogen that can be zoonotically transmitted from

TABLE 2. Classification of 15 samples positive for *Chlamydiaceae* and *Chlamydia*-related bacteria in Ghanaian children

Child no.	Age	Sex	Temperature (°C)	Symptoms	23S rRNA CT	CT	16S rRNA: Sequence identity with first known organism in NCBI database (accession no.)	16S rRNA: Sequence length (bp)	Classification
Febrile with respiratory tract symptoms									
1	2 years	M	38.3	LRTS (cough), URTS (blocked nose)	32.1	33.2	100% *C. pneumoniae* IOL207 (Z49874)	206	*C. pneumoniae*
2	1 year	F	39.4	LRTS (cough), URTS (runny nose)	ND	34.9	100% *C. pneumoniae* IOL207 (Z49874)	206	*C. pneumoniae*
3	1 year	F	39.4	LRTS (X-ray: perihilar infiltrates), no URTS	33.6	34.4	100% *C. pneumoniae* (LN849050)	135	*C. pneumoniae*
4	1 year	F	40	LRTS (cough, nose flaring), URTS (blocked nose, runny nose)	ND	34.5	91.3% *Candidatus*, Syngnamydia venezia strain Pi3-2, partial sequence (KC182514)	207	New family
5	4 years	M	38.7	LRTS (cough), no URTS	32.8	32.2	100% *C. pneumoniae* (LN849050)	205	*C. pneumoniae*
6	3 years	M	39.6	No LRTS, URTS (runny nose)	ND	26.9	100% *C. pneumoniae* IOL207 (Z49874)	108	*Chlamydia*
Fever-free control without respiratory tract symptoms									
7	1 year	F	36.9	NA	ND	34.4	95.9% *Protochlamydia naegleriophila* genome assembly PNK1, chromosome: cPNK (LN879502)	97	*Protochlamydia*
8	1 year	F	36.5	NA	ND	34.2	95.2% *Protochlamydia naegleriophila* strain KNic, partial *sequence* (NR_115817)[a]	146	*Parachlamydiaceae*
9	10 years	M	37.3	NA	31.5	31.2	100% *C. pneumoniae* IOL207 (Z49874)	176	*C. pneumoniae*
10	9 years	M	37	NA	37.2	34.2	90.2% *C. pecorum* (NR_121750)	205	New family
11	11 months	M	37.4	NA	34.6	34.2	100% *C. trachomatis* (HE605380)	176	*C. trachomatis*
12	1 year	F	36.7	NA	22.1	31.5	100% *C. pneumoniae* IOL207 (Z49874)	205	*C. pneumoniae*
13	1 year	F	36.6	NA	23.3	32.8	100% *C. pneumoniae* IOL207 (Z49874)	204	*C. pneumoniae*
14	7 years	M	36.2	NA	34.4	35.3	ND	NA	*Chlamydiaceae*
15	1 year	M	36.9	NA	34.4	35.2	ND	NA	*Chlamydiaceae*

Analysis of children for *Chlamydiaceae* and *Chlamydia*-related bacteria using real-time PCR and sequencing, Ghana. Oropharyngeal samples with cycle threshold ≤35 in 16S rRNA pan-*Chlamydiales* real-time PCR were further analysed by 23S rRNA *Chlamydiaceae*-specific real-time PCR and by sequencing 16S rRNA amplicon.
bp, base pairs (if not self-explaining); CT, threshold cycle; F, female; LRTS, lower respiratory tract symptoms; M, male; NA, not applicable; NCBI, National Center for Biotechnology Information; ND, not done; URTS, upper respiratory tract symptoms.
[a]We obtained two different sequences with forward and reverse primers, both exhibiting 95.2% sequence identity with different BLAST hits; BLAST hit obtained with reverse primer shown. With forward primer, we also obtained sequence assigned to *Parachlamydiaceae* family, but best-known hit was *Neochlamydia* sp. (LN995859).

livestock to humans [25]; this new lineage, however, is clearly distinct from *C. pecorum*. In a healthy 11-month-old boy, we detected *C. trachomatis,* indicating an asymptomatic infection transmitted from a genitally infected mother during vaginal birth.

A recent Swiss report revealed a lack of *Chlamydia*-related bacteria in adult patients with community-acquired pneumonia [8]. In line with this report, we found only one novel *Chlamydia*-related species in a symptomatic child: a febrile 1-year-old girl with a body temperature of 40°C with upper and lower respiratory tract symptoms. The chlamydial species belonged to a novel family that shared 91.3% sequence identity in the 16S rRNA gene with *Candidatus* Syngnamydia venezia, a species related to *Simkania* [26]. In our study, we did not detect any *Simkania negevensis* DNA. This is in accordance with studies that did not observe an association between the presence of *S. negevensis* and respiratory tract infections in children [3,27] and that did not confirm the earlier work of Kahane et al. [28]. In contrast to Swiss children, who were found to be commonly exposed to *Parachlamydiaceae* [3], we found members of this *Chlamydia*-related family in only two healthy Ghanaian children, although none in the symptomatic group. In summary, our results indicate that both *Chlamydiaceae* and *Chlamydia*-related bacteria do not appear to be associated with significant pathogenicity in the Ghanaian children we investigated.

This study provides novel insight into the prevalence of *Chlamydiales* in children from West Africa. Moreover, we have gained a broader view of the spectrum of *Chlamydiales* in respiratory tract infections as well as in healthy children. Our findings of two new families further corroborate that the diversity of species belonging to the *Chlamydiales* order is still not fully established. Further research is needed to precisely locate the role of *Chlamydiaceae* and particularly of *Chlamydia*-related bacteria as aetiologic agents in respiratory tract infections.

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Conflict of interest

None declared.

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